1352-Pos Board B262
How Do Peptides and Drugs Bind to the Peptide Transporters?
Philip W. Fowler, Simon Newstead, Mark S.P. Sansom.
The peptide transporters (TC 2.A.17) are members of the Major Facilitator Superfamily (MFS), the largest family of membrane protein transporters. PepT1 couples the transport of di- and tri-peptides across the lining of the gut to the proton electro-chemical gradient and has also been shown to be responsible for the uptake of several drugs, e.g. the beta-lactam antibiotics. How these MFS transporters can bind and transport both peptides and a wide range of drugs is not known. The occluded structure of PepTSo [1], a bacterial homologue of PepT1, suggests that the peptide transporters can transport such a wide range of substrates because the putative binding site not only contains an electrical dipole but is also surrounded by large, amphipathic residues, notably tyrosine, that can adapt to a wide range of sidechains and chemical groups. We shall test these hypotheses using a combination of unbiased and free energy simulations using molecular dynamics. Any transported substrate must bind to the occluded state as well as the outward-facing state, otherwise it may merely be an inhibitor. If successful, we hope to reveal not only insights that will allow the bioavailability of drug candidates to be improved by targeting them for transport by PepT1 but also how these membrane proteins transport molecules by changing their conformation.
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1353-Pos Board B263
The Na^+-Ca^{2+} Exchanger is Required for Sinoatrial Node Pacemaker Activity in Murine Myocardium
Sabine Groenke, Eric D. Larson, Rui Zhang, Haruko Nakano, Maria C. Jordan, Kenneth P. Roos, Atsushi Nakano, Catherine Proenza, Kenneth D. Philipson, Joshua I. Goldhaber.
To determine whether pacemaker activity in the murine sinoatrial node (SAN) requires Na^+-Ca^{2+} exchange (NCX1) activity, we used cre/loxP technology to generate mice with an atrial-specific knockout (KO) of NCX1 using a sarcolipin promoter. NCX1 atrial KO mice live to adulthood. At 13 weeks, the heart to body weight ratio is increased in KO (5.5 ± 0.1 (n = 20)) versus wild type (WT, 3.9 ± 0.1 (n = 18); p < 0.001). Atria are enlarged with thin walls and show no evidence of NCX1 (including the SAN) on immunostaining. By echocardiography, the left ventricle of the KO is dilated (end diastolic diameter 4.8 ± 0.2 mm in KO (n = 6); 3.6 ± 0.2 mm in WT (n = 4); p = 0.002), and there is a trend towards reduced fractional shortening. The heart rate is markedly lower in KO (362.9 ± 71.2 bpm, n = 6) compared to WT (524.9 ± 56.9 bpm, n = 4, p = 0.005). ECG in KO reveals a junctional rhythm with no identifiable atrial activity (no P waves); this was confirmed using epicardial electrodes in Langendorff-perfused hearts. KO atria loaded with the Ca^{2+} indicator Fluo3-AM exhibit no spontaneous activity (in contrast to the spontaneous transients in WT), but atrial Ca^{2+} transients can be elicited by external pacing. In patch clamped SAN cells isolated from KO mice, there is no NCX activity in response to caffeine-induced SR Ca^{2+} release. L-type Ca^{2+} current is decreased in KO by ~50% but there is no difference in funny current (I_f) between WT and KO. We conclude that NCX1 is required for pacemaker activity in the SAN.

1354-Pos Board B264
Dynamics of P-Glycoprotein: A Fluorescence and Disulfide Cross-Linking Approach
Brandy Verhalen, Stefan Ernst, Michael Börsch, Stephan Wilkens.
P-glycoprotein (Pgp), a member of the ABC transporter family, couples ATP hydrolysis to efflux of hydrophobic molecules including drugs used in chemotherapy. Here we used fluorescence resonance energy transfer (FRET) spectroscopy to delineate the structural rearrangements the two NBDs are undergoing. Cysteines introduced into equivalent regions in the N- and C-terminal NBDs of cysteine-less mouse Pgp were labeled with fluorescent dyes for ensemble and single molecule FRET (smFRET) spectroscopy with lipid-reconstituted Pgp. In ensemble fluorescence experiments, adding substrate and/or nucleotide increased the FRET efficiency for all mutants to varying degrees, suggesting that the two NBDs approach one another upon substrate binding. Analysis of smFRET data suggests that the NBDs of Pgp alternate between at least two major conformations, a low and a high FRET state, during verapamil stimulated ATP hydrolysis. Vanadate inhibition shifted the population toward the high FRET state while the Pgp inhibitor cyclosporin resulted in a shift towards the low FRET state. Furthermore, we have utilized direct disulfide cross-linking to test whether complete dissociation of the NBDs is required for ATPase stimulation by bulky drug molecules. Tethering the two NBDs together at their C-terminal ends did not abolish drug stimulated ATP activity, indicating the motion the NBDs are undergoing is mainly on the level of the catalytic sites, with the C-terminal ends of the NBDs acting as a hinge. Taken together, the data provide an indication as to what the nature and magnitude of the structural rearrangements are that Pgp is undergoing during the catalytic cycle.

1355-Pos Board B265
Determination of the Na^+/Glucose Cotransporter (SGLT1) Turnover Rate using the Ion-Trap Technique
Jean-Philippe Longpré, Jean-Yves Lapointe.
The Na^+/glucose cotransporter (SGLT1) is a membrane protein that couples the transport of 2 Na^+ ions and 1 glucose molecule using the ''alternating access mechanism''. This mechanism involves the cotransporter oscillating between two conformations characterized by intracellular versus extracellular accessibility to the binding sites. The turnover rate (TOR) is the number of complete cycles that each cotransporter performs per second. It is a crucial parameter to establish since it can be used to estimate the number of cotransporters present and it sets a lower limit for the slowest rate constant involved in the cotransport mechanism (conformational changes, binding and de-binding reactions). In this study, we obtained an independent estimate of the TOR for human SGLT1 expressed in *Xenopus laevis* oocytes using a new approach: the Ion-Trap technique (ITT, Blanchard MG. *et al.* AJP 2008;295(5):C1464-72.) where an extracellular ion-selective electrode of large diameter is used to detect the quantity of ions that are rapidly taken up by the cotransporter (within 20 ms) when the binding sites are suddenly exposed to the extracellular solution by application of a negative membrane potential. Taking advantage of the fact that hSGLT1, in the absence of Na^+, can cotransport glucose with protons, we used a pH electrode to determine a TOR of 8.00 ± 1.3 s^{-1} in the presence of 35 mM α-methyl-glucose (αMG) at −150 mV (pH 5.5). This can be used to calculate a TOR of 13.3 ± 2.4 s^{-1}, for the same group of oocytes, under near V_{max} conditions i.e. in the presence of 90 mM Na^+ and 5 mM αMG. Under these circumstances, the average cotransport current was −1.08 ± 0.61 μA (n=14) and this activity was generated by an average of 3.6 ± 0.7 $\times 10^{11}$ cotransporter molecules per oocyte.

1356-Pos Board B266
Alternating Access Mechanism in the Lactose Permease Derived from its Internal Symmetry
Sebastian Radestock, Lucy R. Forrest.
The lactose permease (LacY) is the prototype of the major facilitator superfamily (MFS) of transporters, the largest evolutionarily-related collection of secondary transporters. The substrate transport mechanism of MFS proteins has long been postulated to involve alternating accessibility of the substrate binding site from either surface of the membrane. Existing structural data for LacY reveal a state in which the substrate is exposed to the cytoplasm, but occluded from the periplasm. To elucidate the conformational changes that open the permeation pathway, we examined the internal symmetry of the LacY structure. Our analysis revealed two sets of topologically-inverted repeats. Following ideas from our earlier studies on LeuT and GltPh, a model of the outward-facing conformation of LacY was built by exchanging the conformations of the repeats. The resulting model exhibits all required properties of an outward-facing conformation, namely closure of the binding site residues from the cytoplasm and exposure to the periplasm. Furthermore, the model agrees with a large amount of experimental data. Analysis of differences between the two states suggests a role for conserved sequence motifs in aiding the occlusion of the central pathway. In addition, interactions between pore-lining helices may be conducted to the full conformational change by movements of peripheral helices. Finally, predicted repairing of critical salt-bridging residues in the binding sites agree with previous proposals. Thus, we have generated a reliable atomistic model of the outward-facing state of LacY, using only its internal symmetry, and providing a model for the conformational change of all MFS transporters.

1357-Pos Board B267
Functional Evaluation of Autism-Related Mutations in the Na+ (k+)/ H+ Exchanger NHE9
Kalyan C. Kondapalli, Anniesha Hack, Maya Schushan, Nir Ben-Tal, Rajini Rao.
A number of neurological disorders including autism, have been linked to mutations in a subgroup of intracellular $Na^+(K^+)/H^+$ exchangers, which include NHE6 and NHE9 that localize to recycling and late endosomes respectively. These are thought to mediate the electrically silent counter transport of cations with protons. Yeast ortholog, Nhx1, has been shown to regulate endosomal pH and control vesicular trafficking. Here, we examine the functional consequence of missense mutations in NHE9 associated with autism, using yeast as a first approach. Further, we extend our observations from yeast to a glial cell model, providing the ***first insight into the neurobiological basis of NHE dysfunction***. Previously, a 3-dimensional model of the human NHE1 was constructed using the crystal structure of NhaA, Na^+/H^+ antiporter from *Escherichia coli*, as template.

As the endosomal NHE display significant similarity to NHE1, we used the NHE1 model as a template and used a unique modeling approach to model these other human transporters. Three NHE9 mutations (S438P, L236S, V176I) could be readily extrapolated to yeast Nhx1 from sequence conservation. We used site-directed mutagenesis to replace the yeast residues with the human equivalent, as well as the disease-associated mutation. Mutants were expressed in *nhx1* deletion background and evaluated for pH, salt and trafficking phenotypes. While S43P and L236S mutations led to loss of function phenotypes in yeast, unexpectedly, V167I had no discernable effect. The latter may reveal isoform specific functions that remain to be identified or may call to question the functional contribution to the disease phenotype. In a parallel approach, these NHE9 mutations were expressed in glial cells for functional evaluation in a mammalian cell model. We observed altered trafficking and surface expression of glutamate transporters, consistent with elevated glutamate levels reported in patient brains.

1358-Pos Board B268

Substrate and Inhibitor Binding to a Glutamate Transporter Homologue

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Glutamate transporters tightly control extracellular glutamate concentration by pumping the transmitter into neurons and glia. Active transport is achieved by coupling the energy of the transmembrane ionic gradients to the conformational changes of the transporter, which alternates between outward- and inward-facing states with the substrate-binding site accessible from the extra- and intracellular solutions, respectively. In order to concentrate the substrate into the cytoplasm, the transporter's binding site must exhibit a high affinity from the outside and a low affinity from the inside. To better understand this change, we have characterized the binding properties of a prokaryotic homologue of the glutamate transporters, GltPh, by means of isothermal titration calorimetry. We designed double cysteine mutants that, upon cross-linking, lock the transporter in either the outward- or the inward-facing state and measured the thermodynamic binding parameters of the substrate aspartate and a competitive inhibitor TBOA. Surprisingly, the free energies of aspartate binding to the outward- and inward-facing states of the transporter are very similar at 25 °C, although the enthalpic and entropic contributions differ significantly. GltPh affinity for aspartate depends steeply on the concentration of sodium ions (Na), consistent with binding of ~3 Na being thermodynamically coupled to binding of each substrate molecule. In contrast to the substrate, TBOA shows significantly lower affinity for the inward-facing state compared to the outward-facing state. In both states, TBOA exhibits a weaker sodium-dependence with an apparent number of coupled Na near 1. Our results show that in the absence of a sodium gradient, GltPh binds aspartate and Na with the same affinity in the outward- and inward-facing states. Therefore, the higher substrate affinity on the extracellular side of the membrane is determined by the higher Na concentration in the extracellular space compared to the cytoplasm.

1359-Pos Board B269

Formation of a Leaky State and Molecular Mechanism of Water Co-Transport in Secondary Transporters

Jing Li, Emad Tajkhorshid.

Accumulating experimental evidence suggesting water co-transport and even ion leak currents in specific secondary transporters continues to challenge the principles of the prevailing alternating-access mechanism in this class of transporters. In the alternating-access model, transporter could only expose the central binding site to one side of the membrane, and the ions and the substrate are coupled across the membrane through the carrier-mediated style, which is difficult to explain the high water to solute transport stoichiometry in water co-transport and transport-independent leak current in recent experimental observations. However, the precise mechanisms underlying these observations are still in debate and direct structural evidence of how water and ions might be co-transported along with the substrate is still lacking. We have used extended molecular dynamics simulations of a secondary transporter (vSGLT) for which water co-transport has been well documented in order to investigate the molecular mechanism of the phenomenon. The protein has been simulated in the membrane and initiated from the inward-facing crystal structure in various bound states. The simulations capture a leaky state in which a continuous water channel is formed within the lumen of the transporter, resulting in the accessibility of the central substrate-binding site to solutions at both sides of the membrane simultaneously. During the performed 200-ns simulations, hundreds of water molecules completed full permeation events respectively in either direction. Further structural analysis and comparative investigation identify a gating network as the main mechanism controlling the formation of the leak state, which probably decides the distinct water-cotransport permeability in specific secondary transporters. The leak state captured in our simulations provides novel and deeper insight to the mechanism of transport cycle, and reshape the definition of transporter.

1360-Pos Board B270

Water Permeation Through the Sodium-Dependent Galactose Cotransporter VSGLT

Seungho Choe, John M. Rosenberg, Jeff Abramson, Ernest M. Wright, Michael Grabe.

It is well accepted that cotransporters facilitate water movement by two independent mechanisms: osmotic flow through a water channel in the protein and flow driven by ion/substrate cotransport. However, the molecular mechanism of transport-linked water flow is controversial. Some researchers believe that it occurs via cotransport, in which water is pumped along with the transported cargo, while others believe that flow is osmotic in response to an increase in intracellular osmolarity. In this report, we present the results of a 200-ns molecular dynamics simulation of the sodium-dependent galactose cotransporter vSGLT. Our simulation shows that a significant number of water molecules cross the protein through the sugar-binding site in the presence as well as the absence of galactose, and 70-80 water molecules accompany galactose as it moves from the binding site into the intracellular space. During this event, the majority of water molecules in the pathway are unable to diffuse around the galactose, resulting in water in the inner half of the transporter being pushed into the intracellular space and replaced by extracellular water. Thus, our simulation supports the notion that co-transporters act as both passive water channels and active water pumps with the transported substrate acting as a piston to rectify the motion of water.

1361-Pos Board B271

Dynamics of the Outward and Inward Facing States of the Serotonin Transporter

Saher A. Shaikh, Emad Tajkhorshid.

The serotonin transporter (SERT), a member of the neurotransmitter:sodium symporter (NSS) family, is responsible for clearing neuronal synapses of serotonin, and a major drug target in psychopharmacology. Since no structures have been reported for SERT, structural information is deduced from the crystal structure of LeuT, a prokaryotic NSS homologue. SERT is believed to function by alternating between outward-facing (OF) and inward-facing (IF) states, hence a description of these and intermediate states is vital to the understanding of its transport cycle. To date, studies of SERT structure and dynamics have been restricted to models of the OF state, that in which LeuT is crystallized. We have generated models of substrate/ion-bound as well as free states of SERT, using the OF crystal structure, and our recently published IF models of LeuT. We performed comparative modeling for model generation, docking to position the substrate and extensive optimization of the protein sidechains to generate these models. These SERT models were then simulated in the presence of membrane, water and ions, to characterize the dynamics of the transporter in different states. The simulations reveal differential water permeation behavior among the OF and IF states, and are discussed in view of the known channel-like behavior in SERT. Ion binding patterns hint at a putative binding site for K+, which is known to assist in SERT function. We describe major differences in local and global conformational behavior between the OF and IF states, which provide clues to the transition between these states, and the transport cycle. To our knowledge, these studies provide the first description of dynamics of SERT in the IF state, and a comparative view of the dynamics of the OF and IF as well as substrate/ion bound and free states of SERT.

1362-Pos Board B272

Mitochondrial Membrane Potential in Living Neurons Measured by Flim

Elena E. Pohl, Jutta Schueler, Anne Rupprecht.

Uncoupling protein 4 (UCP4) is an inner membrane mitochondrial protein with localisation in neurons and astrocytes (1). In contrast to UCP1 and UCP2 (2-4), no UCP4 - mediated proton transport activity was demonstrated. To gain insight into the protein function we used fluorescence lifetime imaging (FLIM) for the analysis of mitochondrial membrane potential (MMP). The method provides information about the local fluorophore environment. We first evaluated several probes for MMP measurements including rhodamine dyes, JC-1 and TMRM in living cells. MMP changes were then detected by FLIM in neurons after addition of the artificial uncoupler CCCP or respiratory chain inhibitors (rotenone, antimycin A). The putative UCP activator 4-hydroxy-2-nonenal did not significantly alter membrane potential.

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